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Polarized neutron in structural biology – present and future outlook

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Abstract

Hydrogen has a strong polarization-dependent neutron scattering cross section. This property has been exploited in the study of soft matters, especially biological macromolecules. When a polarized neutron beam is scattered off a polarized hydrogenous sample, the otherwise large hydrogen incoherent cross section is drastically reduced while the coherent signal is significantly increased. Past experiments have demonstrated the potentials and benefits of polarized neutron scattering from soft materials. The main technical challenge of polarized neutron scattering from biological matters lies at sample polarization. Dynamic nuclear polarization is a proven yet rather sophisticated technique. Its complexity is one of the main reasons for the technique's slow adoption. The future of polarized neutron scattering in biology may rest largely in neutron protein crystallography. Polarization of protein crystals is much easier to accomplish, since protein crystals are typically rather small (<<1mm) and only require small and easy- to-operate polarization apparatuses. In addition, the high resolution nature of neutron protein crystallography means that we will be able to study individual atoms using the polarized neutron scattering technique.

Selection and peer-review under responsibility of the Organizing Committee of the 9th International Workshop on Polarised Neutrons in Condensed Matter Investigations

Keywords: Polarized Neutron Scattering, Dynamic Nuclear Polarization, DNP.

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1. Introduction

Hydrogen makes up a large part of atoms in biological molecules. In proteins, about half of all atoms are hydrogen, many of which are functionally important to the protein. In protein kinase, for example, unlocking structural secrets of hydrogen atoms and bound water molecules near the active catalytic site is crucial to the understanding of the kinase's functional mechanism. One of the most valuable structural tools today is X-ray diffraction, made possible by decades of advances in synchrotron radiation. Many tens of thousands of atomic resolution protein structures have been deposited into the protein data bank today[1]. Nonetheless, since X-rays are not sensitive to light elements, the majority of these deposited structures contain little or no information on hydrogen atoms. In this regard, neutron scattering plays a unique role in structural biology. Even with typical beam intensities many oders of magnitute weaker than synchrotron radiations, neutron protein crystallography has been able to provide the missing information on hydrogen structures in proteins [2]. Due to the fact that neutron scattering length has no correlation to the atomic number of the scattering element and it is isotope sensitive, neutron scattering and diffraction techniques have become ever more important to structual biology.

The scattering of neutrons by hydrogen atoms, or protons, depends strongly on the spin states of the protons and neutrons. Consequently, when unpolarized, a large part of the neutron scattering from hydrogen atoms is incoherent. The coherent and incoherent neutron scattering cross-section of protons, for example, is ~1.8 and 80 barns, respectively. This large incoherent scattering manifests itself as background and it decreases experimental signal-to-noise ratio. To overcome hydrogen incoherent scattering, hydrogen/deuterium (H/D) substitution is often used. The incoherent neutron scattering cross-section of deuteron is 2.05 barns only. Its coherent cross-section is 5.59 barns. A simple H/D substitution process is to soak protein crystals in deuterated medium for an extended period of time. Crystal waters and the majority of exchangeable protons in the protein will then be exchanged to deuterium. However, this process does not replace those hydrogens that are tightly bound in the protein. A more recent development is protein perdeuteration. Proteins are expressed in bacteria grown on deuterated media. The thus grown proteins are then purified and grown into crystals [2]. However, perdeuteration is still expensive and growing deuterated crystals is not always as easy as compared to nondeuterated ones.

Polarized neutron scattering from polarized biological macromolecules aims at harvesting the large spin dependent neutron cross-section. In general, the scattering length of a neutron from a nucleus with the spin **I** can be written as $b = b_0 + b_1(\mathbf{I}\cdot\mathbf{s})$. **s** is the neutron spin vector. b_0 and $b_1$are the spin independent and spin dependent parts of the scattering length, respectively. For protons, $b_0 = -3.74$ *fm*, $b_1 = 58.2$ *fm*. The total polarization dependent coherent and incoherent neutron scattering cross-sections are [3]

$$\sigma_{coh} = 4\pi[b_0^2 + P_n P_1 \cdot I b_0 b_1 + \tfrac{1}{4} P_1^2 \cdot I^2 b_1^2] \quad (1)$$

$$\sigma_{inc} = \pi[I(I+1)b_1^2 - P_n P_1 \cdot I b_1^2 - P_1^2 \cdot I^2 b_1^2] \quad (2)$$

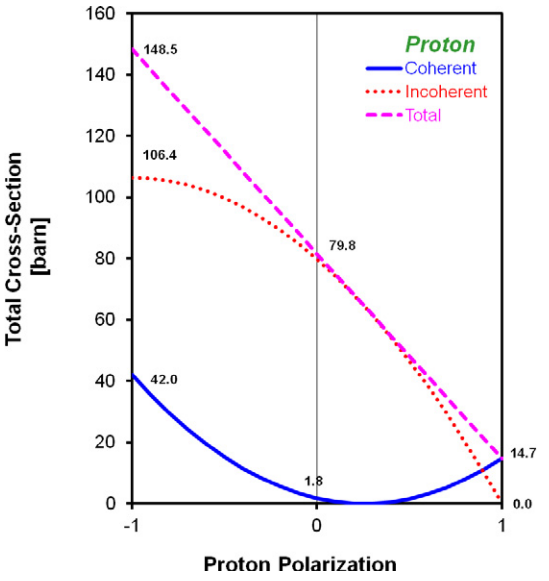

Fig. 1. Neutron scattering cross-sections of hydrogen as a function of the proton polarization. The polarization of the neutrons is assumed to be 100% for the calculations.

P_I and P_n are the polarizations for the nuclei and neutrons, respectively. Fig. 1 plots out these equations for hydrogen as a function of proton polarization. It's evident that the incoherent cross-section vanishes when neutrons and protons are polarized in parallel. In the mean time, the coherent cross-section increases by close to an order of magnitude. It is this simultaneous increase in coherent cross-section and decrease in incoherent cross-section that has given polarized neutron scattering its potential for structural biology.

The main difficulty for conducting polarized neutron scattering from biological samples has been to obtain high sample polarization. Due to the lack of fast relaxation mechanisms in these samples, brute force polarization cannot be used. To achieve high nuclear polarizations, extreme low temperatures and high magnetic fields are required. However, the nuclear relaxation times under such conditions are very long, making the brute force method of biological samples impractical. The only viable option today is to use the technique of dynamic nuclear polarization (DNP). DNP uses microwave pumping to transfer the electron polarization of paramagnetic centers to nuclear spins at low temperatures and high magnetic fields. For biological samples, the paramagnetic centers are typically in the form of EHVA-Cr(V) [4] or TEMPO [5] added to the sample. Typical magnetic fields for DNP are about 2.5-5T and sample temperatures are about 1K or lower.

2. Developments of polarized neutron scattering from biological macromolecules

The majority of polarized neutron scattering from dynamically polarized biological samples developments took place under the leadership of Dr. H. B. Stuhrmann at the GKSS research center, now the Helmholtz Center, in Geesthacht, Germany. Initially, the project focused on overcoming the resolution limit [6] in the contrast variation technique for the study of ribosomal proteins [4]. Ribosome is the cellular protein factory and is one of the most important molecular machineries in cell. Understanding its structure is especially important to molecular and cell biology. Until its crystal structure was resolved by synchrotron radiation [7], ribosome posed a major challenge to structural biology due to its enormous size and complexity. One of the more successful methods of studying the ribosome was using small angle neutron scattering (SANS) in combination with H/D substitution. Selected proteins within the ribosome were deuterated and their spatial separation was determined from small angle neutron scattering experiments. With enough of these protein-protein distances, the locations of all the proteins within the ribosome were then determined using triangulation. This technique was successfully used on the small subunit

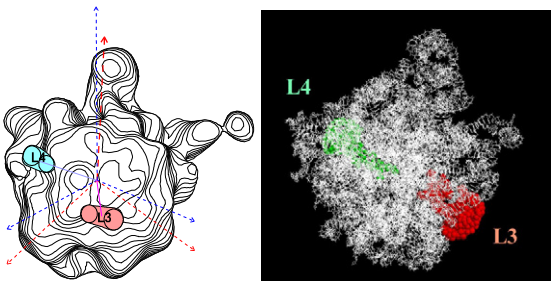

Fig 2. Locations of the L3 and L4 proteins as determined from polarized neutron scattering (left, [11,12,13]) and protein crystallography (right, [7]). The agreement is remarkably good.

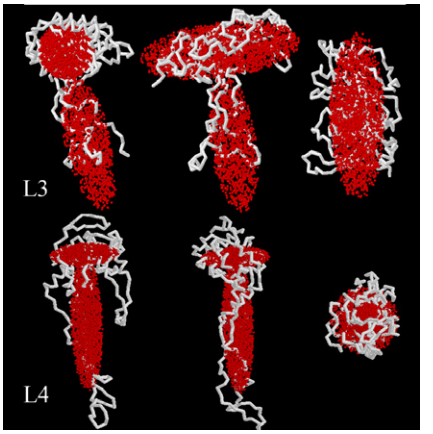

Fig 3. Three perpendicular views of the low resolution structure models for the L3 (top) and L4 (bottom) proteins determined from polarized neutron scattering studies (dots, model data extracted from [11,12,13]) and from their crystal structures (coils [7]). The determination of protein asymmetry would not have been possible if

of prokaryotic ribosome [8]. For the large ribosomal subunit, the same technique was no longer applicable in a straightforward fashion, simply because the large subunit is too big. With its massive molecular weight of 1.5MDa, the large ribosomal subunit contributes immensely to the background scattering signal. The contrast provided by H/D substitution was not sufficient for scattering experiments to determine the protein-protein separations. At the time of this experimental impasse, sample polarization technique using DNP had matured, especially within the high energy physics community [9]. DNP enables polarized neutron scattering from biological samples and allows the overcoming of the contrast limit by H/D substitution.

During its decade of endeavor, Dr. Stuhrmann's group not only demonstrated the feasibility of polarized neutron scattering from biological samples, it also produced many ribosomal structures that were inaccessible to other structural tools at the time [10]. The technique's potential is best demonstrated by the study two ribosomal proteins, L3 and L4 within the large ribosomal subunit [11,12,13]. Using protiated L3 and L4 proteins embedded in deuterated ribosome, polarized neutron scattering experiments were able to determine not only the locations of the proteins within the ribosome (fig. 2), but also the asymmetries of the proteins (fig. 3). Crystal structure of the ribosome [7] that was solved many years later confirmed these results.

Concurrent to these above developments, there were several other efforts on polarized neutron scattering from dynamically polarized samples. Some of these early efforts were at CEA, Saclay France [14]; KEK, Japan [15], and PSI, Switzerland [5]. With the exception of PSI, most of the other works focused on polymers and other soft matters. Today, one of the more active groups in these area is at JAERI, Japan [16,17,18].

3. Current and future work on polarized neutron protein crystallography

Most polarized neutron scattering from dynamically polarized samples experiments use DNP setups that were initially developed for different purposes, mostly for high energy and nuclear physics. Consequently, these equipments are often far from optimized for neutron scattering experiments. They are often very large, highly specialized, and expensive to operate. Many of the scattering experiments were conducted on small angle neutron scattering instruments. Though otherwise a very powerful structural tool, SANS is a low resolution and low information content technique. For structural biology, the eventual goal of most experiments is to obtain atomic resolution structures of the macromolecule under study. Integrating polarized neutrons with protein crystallography is thus the next natural step for neutron structural biology. In addition to being a high resolution structural method, neutron protein crystallography requires samples that are 3-4 orders of magnitude smaller than those in SANS experiments. Protein crystals are commonly <<1mm^3 in volume.

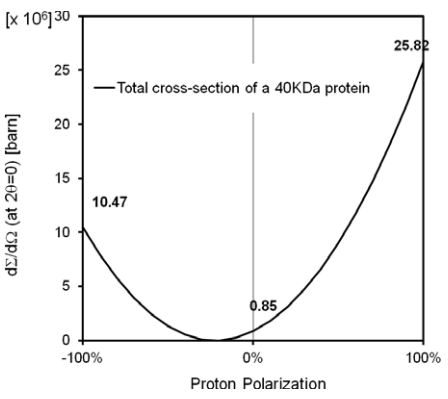

Fig. 4 Total coherent scattering cross-section for a typical sized, 40 kDa protein. Neutron polarization is assumed to be 100%.

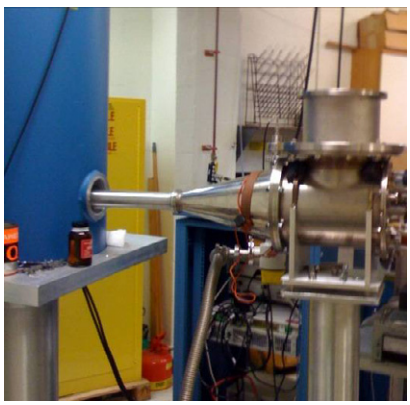

Fig. 5 DNP setup at the SNS for dynamic polarization of protein crystals. The setup uses a 5 T magnet and the 1K refrigerator.

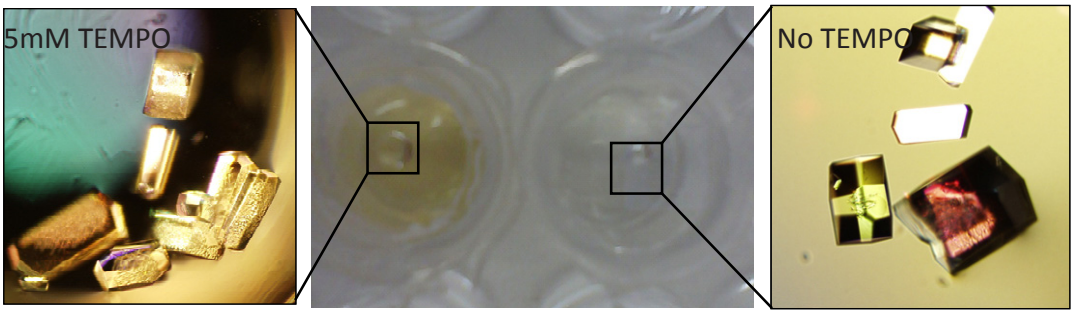

Fig. 6 Lysozyme crystals in 5% NaCl and 50mM Sodium Acetate. Left: with 5mM TEMPO. Both the dish well and the crystal show light yellow. Right: No TEMPO.

The required DNP setup for such small samples is easily built to be small, portable, and inexpensive to operate. Comparing to synchrotrons, neutron protein crystallography is still severely flux limited and thus will benefit greatly from polarized neutrons. Fig. 3 shows the calculated total coherent scattering cross-section of a typical sized protein. The gain in diffraction intensity can be considerable.

In addition to gains in diffraction intensity, polarized neutron protein crystallography will automatically reduce the incoherent background (fig. 1). Furthermore, polarizations of various nuclear species in dynamically polarized samples can be manipulated by NMR signals such that only one desired species, such as hydrogen, is polarized. The selective polarization/depolarization techniques can also be used to create polarized clusters around paramagnetic centers [5,11,13].

At the SNS, we have conducted initial explorative researches on dynamic polarization of protein crystals with the aim of building a foundation for future application of polarization neutron protein crystallography at the SNS. Our experiments try to find the best ways to introduce paramagnetic centers into protein crystals. These paramagnetic centers, or free radicals, act at the polarizing agents for DNP. During decades of developments, several types of free radicals were found to be good polarization agents for polarizing soft matters. They include EHBA-Cr(V) [4], TEMPO [5], and radicals created by electron beam radiation [17,19]. One form of these radicals, the spin label, is of particular interest. Similar to those used in electron paramagnetic resonance studies, spin labels attach themselves to specific sites on the protein surface. Since polarized nuclear spin clusters can be created around the paramagnetic center [5,11,13], the usage of spin labels may offer ways to study specific areas on the protein. Fig. 5 shows the dynamic sample polarization system used for these studies. Several types of protein crystals were tested. One of them is lysozyme. Lysozyme's structure is well known and its crystal can be produced in large quantity. Thus, it provides a good platform for our test. Fig. 6 shows pictures of lysozyme crystals with and without the co-crystallization of TEMPO. The structure of the lysozyme is monitored using table-top X-ray diffractions and solved by molecular replacement with MOLREP [20]. The introduction of TEMPO into the crystal does not seem to have any noticeable effect on the lysozyme structure (fig 7). However, several regions with positive electron density on the surface of lysozyme were observed (fig 8). The density of these regions is much higher than

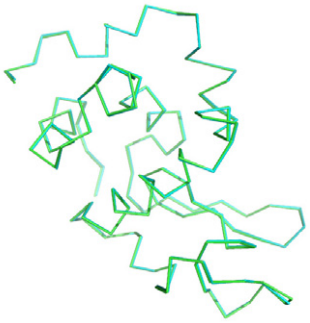

Fig. 7 Superposition of lysozyme crystal structure backbones with (blue) and without (green) co-crystallized TEMPO radicals. The green structure is obtained from the Protein Data Bank (1AZF.pdb, [1]) and is used as the reference. The structure with co-crystallized TEMPO was solved by molecular replacement with MOLREP [20]. The two structures overlap to a high degree such that the blue one is barely visible in the picture. The root mean square of the deviation between the two structures is 0.17 Å.

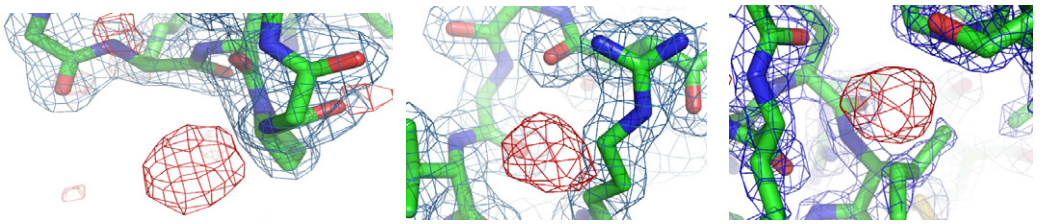

Fig 8. Different regions on the lysozyme surface that have positive electron density. These regions possibly correspond to TEMPO molecules.

typical water molecules. It's possible that these regions represent poorly ordered TEMPO molecules or protein regions oxidized by TEMPO. Even though the form of TEMPO used in these experiments was not specific in interacting with amino acids, under the low pH (4.8) condition that the samples were in, TEMPO may act as oxidants and interact with the protein. Future experiments will focus on spin label forms of the TEMPO, such as 4-(2-Iodoacetamido)-TEMPO, that can bind to cysteine residues on protein surface (Fig 9). Other TEMPO derivatives, such as (3-(5-Fluoro-2,4-dinitroanilino)-PROXYL), can bind to lysine and arginine residues on protein surface and will also be evaluated.

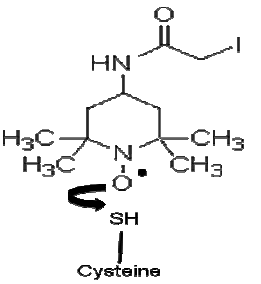

Fig. 9 The 4-(2-Iodoacetamido)-TEMPO forms a disulphide bond with cysteine residues on protein surface.

Initial polarization attempts indicate that these lysozyme crystals can be polarized, though the degree of polarization still needs to be improved (Fig 10). In an ongoing collaboration between the SNS and the University of Virginia, efforts are now under way for a dedicated dynamic sample polarization station for protein crystallography. Our near future goal is make polarized neutron protein crystallography at the SNS a reality and hence greatly enhance the capability of neutron protein crystallography.

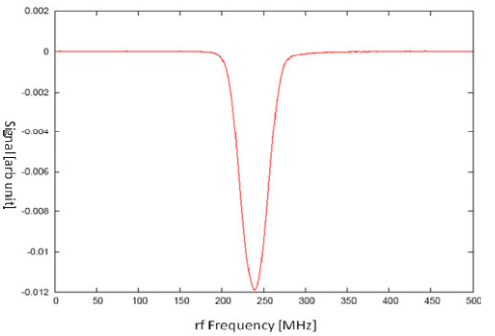

Fig. 10 Proton NMR signal from polarized lysozyme protein crystal. The corresponding polarization is about 30%

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